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Synergistic Effect of Titanium Alloy and Collagen Type I on Cell Adhesion, Proliferation and Differentiation of Osteoblast-Like Cells

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Key Words

Titanium · Osteoblasts · Collagen type I · Focal adhesions · Immunocytochemistry · Rat

Abstract

A number of studies have demonstrated the pivotal role of collagen in modulating cell growth and differentiation. In bone, where the extracellular matrix is composed of approximately 85% type I collagen, cellular interaction with matrix components has been shown to be important in the regulation of the osteoblast phenotype. Preservation or enhancement of normal osteoblast function and appositional bone formation after implant placement represents a strategy that can be useful for the purpose of improving osseointegration. In order to further improve biocompatibility, we combined two known favorable compounds, namely the titanium alloy, Ti6A14V, with type I collagen. We assessed the in vitro behavior of primary osteoblasts grown on both fibrillar collagen-coated and tropocollagen-coated Ti6A14V in comparison with uncoated titanium alloy, using an improved adsorption procedure. As parameters of biocompatibility, a variety of processes, including cell attachment, spreading, cytoskeletal organization, focal contact formation, proliferation and expression of a differentiated phenotype, were investigated. Our results demonstrated for the first time that in comparison to uncoated titanium alloy, collagen-coated alloy enhanced spreading and resulted in a more rapid formation of focal adhesions and their associated stress fibers. Growing on collagen-coated Ti6A14V, osteoblasts had a higher proliferative capacity and the intracellular expression of osteopontin was upregulated compared to uncoated titanium alloy. Type I collagen-coated titanium alloy exhibits favorable effects on the initial adhesion and growth activities of osteoblasts, which is encouraging for its potential use as bone graft material. Moreover, collagen type I may serve as an excellent biocompatible carrier for osteotropic factors such as cell adhesion molecules (e.g. fibronectin) or bone-specific growth factors.

Abbreviations used in this paper

BSA	Bovine serum albumin
ELISA	Enzyme-linked immunosorbent assay
FC	Fibrillar collagen
FITC	Fluorescein isothiocyanate
PBS	Phosphate-buffered saline
UC	Uncoated
TC	Tropocollagen
TRITC	Tetraaminorhodamineisothiocyanate

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Introduction

One of the most common orthopedic and dental implant materials is the metal alloy Ti6A14V. Titanium alloys are well known for their superior mechanical properties and biocompatibility, making them desirable as surgical implant materials. Essential for the biocompatibility of an implant are its growth-enhancing surface properties [Martin et al., 1995], which are a prerequisite for sufficient long-term performance [Davies, 1996]. Implanted material attains and maintains contact with interfacial tissue via its surface. The growth and proliferation of osteoblastic cells on these implants are necessary for osseointegration, which is defined by an initial close apposition of bone and long-term stable bone ingrowth to the alloy surface [Albrektsson and Albrektsson, 1987; Davies, 1996; Davies et al., 1986, 1990]. Cell adhesion, spreading and signaling are complex processes that are dependent on the chemistry of the substrate with which the cells interact [Kieswetter et al., 1996; Shah et al., 1999].

As a consequence, there is an ongoing interest in coating implant alloys with proteins to improve biocompatibility and cell-adhesive properties [Kornu et al., 1996; Volz and Benjamin, 1990]. One strategy to achieve this goal is to precoat implants with cell adhesion molecules. Fibroblast adhesion to metal implants with and without pretreatment of the implants with various cell adhesion molecules has been well documented [Cannas et al., 1988; Curtis and Forrester, 1984; Dejana et al., 1987; Guy et al., 1993; Hayman et al., 1985; Hughes et al., 1993; Hynes, 1992; Klein et al., 1994; Kornu et al., 1996; Lowenberg et al., 1988; Michaels et al., 1991]. Similarly, a number of studies have revealed how osteoblast adhesion to implants influences osseointegration [Kornu et al., 1996; Puleo et al., 1991; Shah et al., 1999; Sinha et al., 1994; Vrouwenvelder et al., 1993]. The most widely used cell adhesion molecule is fibronectin, a major component of serum, but the matrix components laminin, fibrinogen and vitronectin have also been applied. However, the major structural protein of the bone matrix, collagen I, has not been tested so far on titanium alloys for osseointegration.

The present study tested the hypothesis that the biocompatibility of titanium alloy will be promoted after adsorption of type I collagen onto its surface. In bone, cellular interaction with matrix components is important for regulation of the osteoblast phenotype [Andrianarivo et al., 1992; Celic et al., 1998; Traianedes et al., 1993]. Several studies have indicated that type I collagen accelerates the expression of the bone cell phenotype [Andrianarivo et al., 1992; Aronow et al., 1990; Celic et al., 1998; Franceschi and Iyer, 1992; Lynch et al., 1995; Masi et al., 1992; Owen et al., 1990; Traianedes et al., 1993].

According to our hypothesis, collagen coating of Ti6A14V will cause differential osteoblastic cell adhesion due to changes in cell-extracellular matrix interactions. Cell surface receptors involved in cell adhesion, such as integrins, have been implicated in signal transduction and regulation of gene expression. Integrins cluster to form the focal adhesion contact [Albelda and Buck, 1990; Burridge et al., 1988], a complex that includes proteins such as talin, α-actinin and vinculin, anchors intracellular stress fibers and contributes to cell spreading [Green et al., 1995; Shah et al., 1999].

Whereas the individual effects of collagen type I or titanium alloy on osteoblasts are well documented, a study that integrates both variables is still lacking. Therefore, we have assessed the potential of collagen type I coating of titanium alloy to influence a variety of in vitro processes. In this study, the effect of coating titanium alloy with monomeric (tropocollagen) and fibrillar collagen type I was investigated. Primary calvaria-derived osteoblastic cells were used to compare cell adhesion kinetics, including cell attachment, spreading, cytoskeletal organization and focal contact formation, proliferation and expression of a differentiated phenotype.

Materials and Methods

Test Substrates

As substrates for the cell experiments, the following surface states were used: Ti6A14V without coating, Ti6A14V coated with tropocollagen, and Ti6A14V coated with fibrillar collagen. Disks of 16 mm diameter and 2–3 mm thickness consisting of the orthopedic titanium alloy Ti6A14V (ASTM F 136) were used after mechanical polishing and a cleaning procedure (ultrasonic treatment in 1% Triton X-100, acetone and 96% ethanol). The surface showed a variety of unoriented grooves resulting in a mean roughness of 42 nm, which was determined by an atomic force microscope (data not shown).

An improved adsorption method of tropocollagen monomers and fibrillar collagen was used [Rössler et al., 1999, 2000]. Briefly, a stock solution of 1 mg/ml calf skin collagen type I (acid soluble; Fluka, Deisenhofen, Germany) in 0.01 *M* acetic acid was prepared. For coating with tropocollagen, the substrates were exposed to a solution of 0.5 mg/ml collagen I in 0.065 *M* phosphate buffer (pH 6.5) at 4°C for 15 min and subsequently rinsed with deionized H_2O. Fibrillar collagen was obtained by in vitro reconstitution in a solution of 0.5 mg/ml collagen I in 0.065 *M* phosphate buffer (pH 7.0, 37°C). Following homogenization of the solution and the separation of remaining tropocollagen by centrifugation, a suspension of fibrillar collagen (approximately 1 mg/ml) in 0.133 *M* phosphate buffer (pH 7.0) was prepared and allowed to adsorb for 15 min at room temperature. The coated surfaces of the disks were examined by scanning electron

microscopy (Leo, Cambridge, UK) and immunogold staining. Tropocollagen was quantified using ELISA, while Sirius Red staining was employed for collagen fibrils. Prior to cell culturing, the disks were sterilized with 100% ethylene oxide at 42°C. There was no measurable desorption of collagen after exposure of the coated titanium alloy to the cell culture medium for several weeks.

Cell Cultures

Primary osteoblastic cells were obtained from fetal rat calvaria samples by collagenase digestion. The bone samples were dissected in approximately 1-mm^3 fragments after removal of the periosteum. They were washed first with tyrode solution, then with Ham's F12 medium and then digested in 4 ml of collagenase-trypsin solution [137 mg of collagenase type I (Biochrom, Berlin, Germany) and 50 mg of trypsin type III (Sigma, Deisenhofen, Germany) in 10 ml of deionized H_2O containing 8 g of NaCl, 0.2 g of KCl and 0.05 g of $NaH_2PO_4 \cdot H_2O$ per 100 ml of deionized H_2O] at room temperature. Cells from the first 45-min digest were discarded. The second and third 30-min digests (in 4 ml of fresh collagenase-trypsin solution) were centrifuged for 2 min at 580 *g*. The cell pellets were resuspended in 4 ml of Ham's F12 medium containing 20% fetal calf serum. After 10 min of centrifugation at 500 *g,* the cells were plated in full medium, i.e. 12 ml of Ham's F12 medium containing 12% fetal calf serum, 2.3 m*M* Mg^{2+}, 100 IE/ml penicillin and 100 μg/ml streptomycin sulfate. The medium was changed every 3 days. The osteoblast phenotype was characterized by determination of alkaline phosphatase activity, collagen type I synthesis and formation of calcium phosphate deposits. The cells were used for between four and five passages. The cultures were maintained at 37°C in humidifed air and 5% CO_2. For mineralization, the cultures were allowed to grow to confluency (3 days). Then the media were supplemented with 50 μg/ml ascorbic acid and 10 m*M* β-glycerophosphate. Cell viability was assessed by trypan blue dye exclusion.

Application of Cells to Implants

The 16-mm disks were placed into 12-well Falcon culture plates (Becton Dickinson, Heidelberg, Germany). Primary osteoblasts were passaged using a 0.05% trypsin/0.02% EDTA solution and plated carefully on the test substrates at a single-cell density of 10,000 cells/cm^2. The cells were allowed to settle for 2 h in the incubator at 37°C, after which, 2 ml of complete medium was added. The cells were then incubated for different periods of time (see Results). For adhesion assays up to 2 h, nonadherent cells were gently rinsed away with phosphate-buffered saline (PBS), pH 7.4.

Immunocytochemistry

Fixation, permeabilization, blocking and PBS washes were all performed at room temperature. After the specific culture time, the cells were washed with PBS and fixed with 4% formaldehyde for 5 min. The cells were then permeabilized for 6 min in 0.5% Triton X-100 (in PBS) and rinsed with PBS. Nonspecific binding was blocked by incubation in 1% BSA in PBS for 10 min. For demonstration of focal contacts [Plopper et al., 1995], the cells were incubated with a mouse monoclonal antibody against human vinculin (dilution 1/20; BioGenex, Hamburg, Germany), known to cross-react with rat vinculin, followed by FITC-coupled goat anti-mouse antibody (Sigma). To detect filamentous actin of the cytoskeleton, the cells were incubated with TRITC-conjugated phalloidin (0.1 μmol/l; Sigma) for 30 min. The cells were observed using both a conventional fluorescence microscope (Leica, Bensheim, Germany) and a confocal laser scanning microscope (Leica). The confocal laser scanning microscope was used to optically section the cells orthogonally with respect to the substrate surface in order to analyze the three-dimensional relationship between the substrate surface and spreading.

Flow Cytometry

Proliferation. For the proliferation assay, the cells were used from a pool of 3 animals obtained in one preparation. The controls were performed with cells of the same population. Cells in the various phases of their cell cycles were characterized by specific DNA content and assessed by flow cytometry, as follows: after 24, 48 and 72 h of culture on substrates, the cells were detached by treatment with trypsin/EDTA, washed with PBS, and suspended in 70% ethanol. After washing, the cells were incubated with RNase A (1 mg/ml; Becton Dickinson) and stained with propidium iodide (Becton Dickinson). Flow cytometry analysis was performed using a FACScan (Becton Dickinson).

Osteopontin Expression. The expression of osteopontin was measured after 4, 8, 11 and 18 days of culture, when osteoblasts were fully confluent. After 3 days of culture, the media were supplemented with 50 μg/ml ascorbic acid and 10 m*M* β-glycerophosphate. The cells were washed twice with PBS and detached using trypsin/EDTA. After washing, the cells were resuspended at 5×10^6 cells/ml, immediately fixed in 2% (w/v) formaldehyde in PBS for 20 min and centrifuged. Then the cells were resuspended in a washing buffer consisting of 0.5% BSA in PBS. Aliquots of 10^5 cells were used to measure the relative level of osteopontin by flow cytometry. The cells were permeabilized for 20 min using 0.5% (w/v) saponin (Sigma) in washing buffer. They were then washed, centrifuged, resuspended in washing buffer and incubated for 60 min with a mouse anti-rat osteopontin monoclonal antibody ($MPIIIB10_1$, Developmental Studies Hybridoma Bank, University of Iowa, Iowa, USA). Subsequently, the cells were washed twice in PBS containing 0.5% BSA and 0.5% (w/v) saponin and incubated with an FITC-anti-mouse IgG (Sigma) for 60 min at room temperature. The cells were washed again and resuspended in 400 μl of PBS, and analyzed by flow cytometry.

Statistical Analysis

Each experiment was carried out in duplicate and repeated three times with different cell culture preparations. Data are presented as means plus or minus the standard deviation, as wells as the number of experiments (n). Each symbol on the graphs corresponds to the mean obtained with triplicate cultures.

Results

Attachment and Spreading of Osteoblasts on Collagen-Coated Titanium Alloy

In this experiment, we compared osteoblastic cell adhesion kinetics, including cell attachment, spreading, cytoskeletal organization and focal contact formation, on type I collagen-coated and uncoated titanium alloy. The adhesion of osteoblasts was examined using immunocytochemistry for vinculin to analyze focal adhesion formation and incubation with phalloidin for the visualization of actin organization and distribution (stress fibers). A

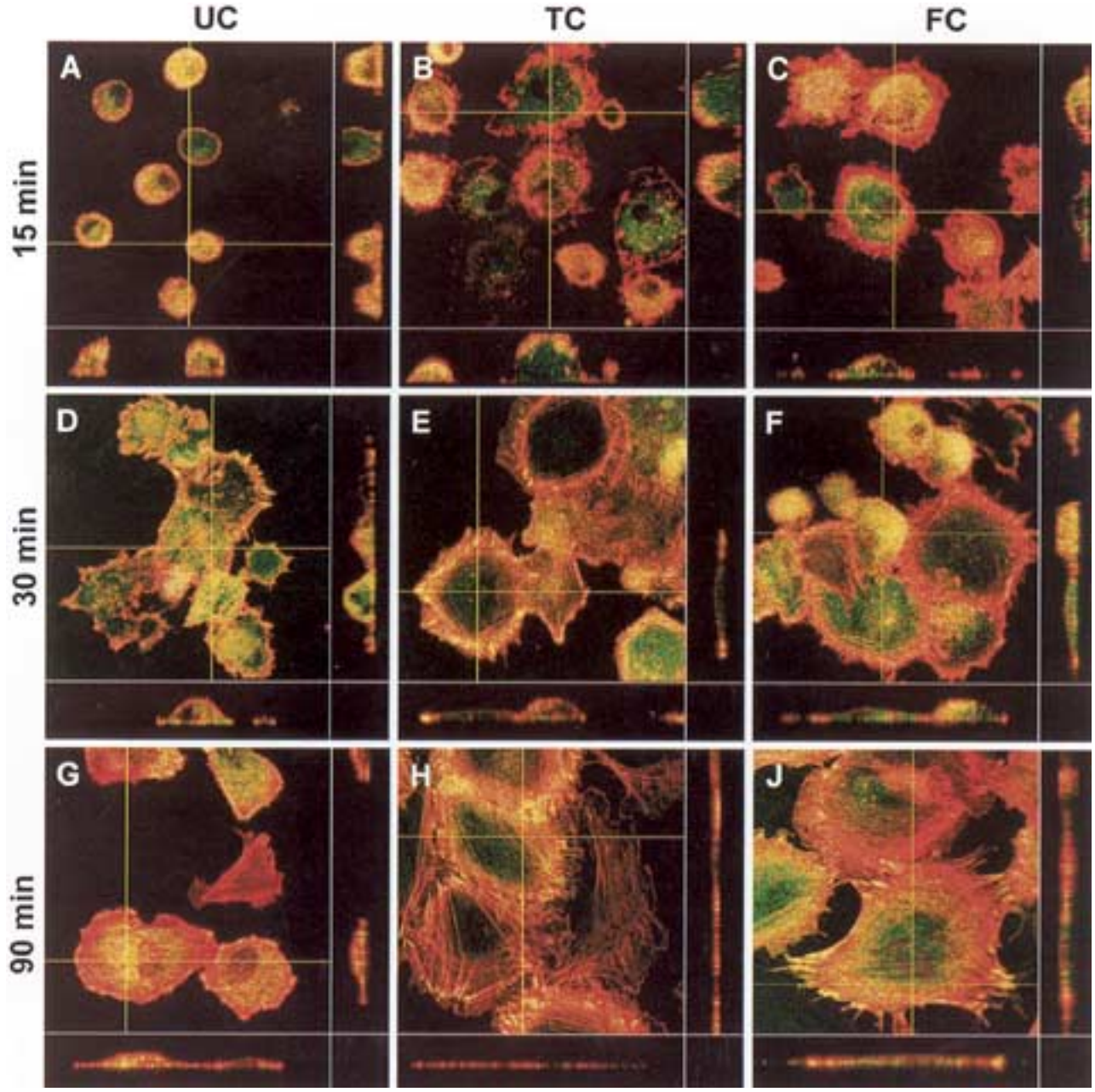


Fig. 1. Characterization of osteoblast initial adhesions. Osteoblasts were plated for 15 min (**A–C**), 30 min (**D–F**) and 90 min (**G–J**) on Ti6A14V disks, which were uncoated (UC), coated with tropocollagen (TC) or coated with fibrillar collagen (FC). The cells were then double labeled for actin stress fibers (phalloidin; red fluorescence) and vinculin (green fluorescence). Optical sectioning (see plane of section) along the indicated x-z and y-z planes shows the cell peak on the surface. Individual field widths: 100 μm.

time course for initial cell adhesion was conducted at periods of 15, 30 and 90 min, as well as 6 and 24 h. We observed that osteoblasts adhered similarly after 2 h to all surfaces. Few, if any nonadherent osteoblasts were present in the culture medium, suggesting that the disks did not cause extensive toxicity.

During the first 2 h, the morphological appearance of the osteoblasts grown on uncoated and coated titanium alloy varied considerably. Using confocal laser scanning microscopy, we observed that, during the first 90 min of contact with the substratum, the degree of spreading of primary osteoblastic cells, the cytoskeletal organization

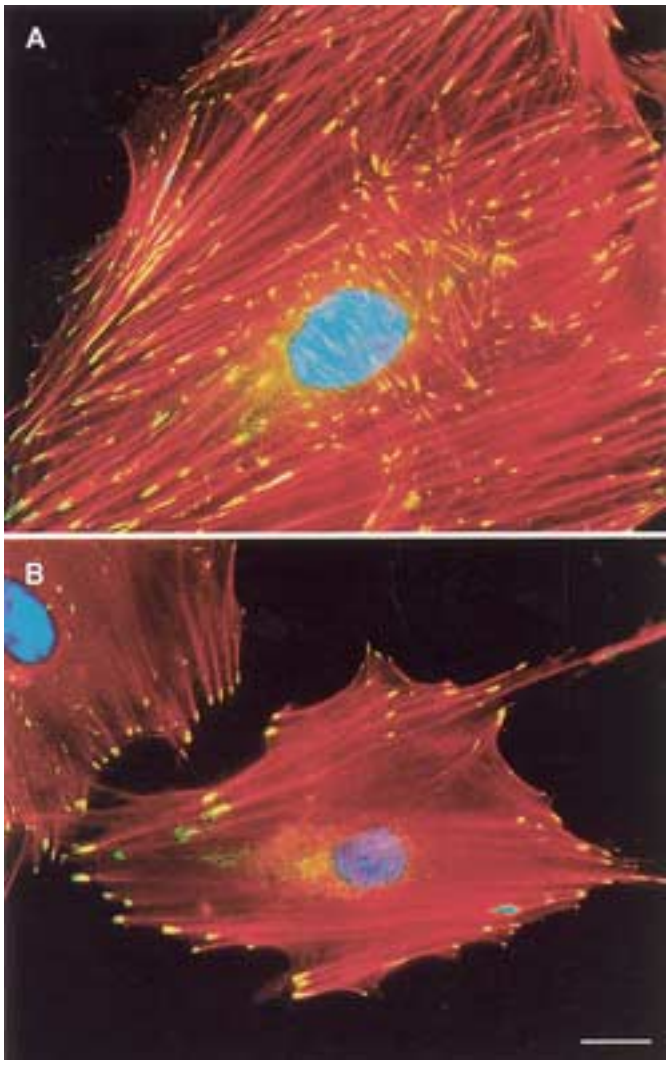


Fig. 2. Morphology of osteoblasts cultured for 6 h on uncoated Ti6A14V disks, which display no difference to those on other surface states at this time. Cells were labeled for actin stress fibers (red), vinculin (green) and DNA (DAPI; blue). Scale bar (shown in **B**) for **A** is 4 μm, for **B** 10 μm.

and focal contact formation differed greatly among substrates.

Initially, round osteoblasts attached to the substrates, with subsequent spreading of the cells after 15 min (fig. 1A–C). F-actin of the rounded cells was generally diffusely distributed, with circumferential labeling near the cell plasma membranes. Osteoblasts attached to the uncoated surface but failed to spread (fig. 1A). However, on tropocollagen-coated (fig. 1B) and fibrillar collagen-coated (fig. 1C) titanium alloy, the osteoblasts appeared to be flattened. The spreading morphology of osteoblasts on tropocollagen was similar to that on fibrillar collagen. After 30 min, the osteoblasts were more completely spread and F-actin was diffusely distributed, with circumferential banding near the edges of the cells (fig. 1D–F). By this time, streaked areas of vinculin became obvious in osteoblasts adhering to tropocollagen-coated (fig. 1E) and fibrillar collagen-coated (fig. 1F) titanium alloy. Cells cultured on uncoated titanium alloy demonstrated less extensive spreading (fig. 1D). After 90 min, osteoblasts on collagen-coated titanium alloy were spread and polygonal, and thin microfilament bundles were visible (fig. 1H, J). In contrast to uncoated surfaces (fig. 1G), cells grown on collagen-coated titanium alloy demonstrated apparently greater spreading, with cellular extensions in multiple directions. In the well-spread osteoblasts cultured on coated surfaces, multiple distinct stress fibers extended across the entire length of the cells, and occasionally the cytoplasm was encircled by a band of stress fibers. The discrete, streak-like focal contacts, observed after 90 min on collagen coating, were generally found at the termini of stress fibers near the cell plasma membranes. The amount of focal contacts increased with time. Conversely, an intense and homogeneous staining with circumferential banding near the cell plasma membranes was seen in osteoblasts cultured on uncoated surfaces for 90 min. However, actin cytoskeletal reorganization was prominent in cells cultured on collagen-coated titanium alloy, with stress fibers arranged throughout the cell body, whereas, on uncoated surfaces, actin filaments were sparse and localized primarily near cellular extensions. In general, after 6 h, the well-spread cells exhibited polygonal arrays of stress fibers, and this spreading sequence was similar on all materials tested. The cells had distinct, parallel-running actin filaments along the long axis of the cell (fig. 2).

In summary, the degree of cytoskeletal organization was enhanced on tropocollagen-coated and fibrillar collagen-coated titanium alloy compared with uncoated titanium alloy, correlating with better focal contact formation during the first 2 h.

Proliferation of Osteoblasts on Collagen-Coated Titanium Alloy

Flow cytometric analysis of DNA-associated propidium iodide was used to evaluate cell proliferation on collagen-coated titanium alloy. For each test, 10,000 cells were analyzed. Figure 3 shows a representative histogram of cells grown on titanium alloy, in which the first main peak corresponds to cells with a G0/G1 DNA content (2n DNA level), and the second, whose fluorescence intensity

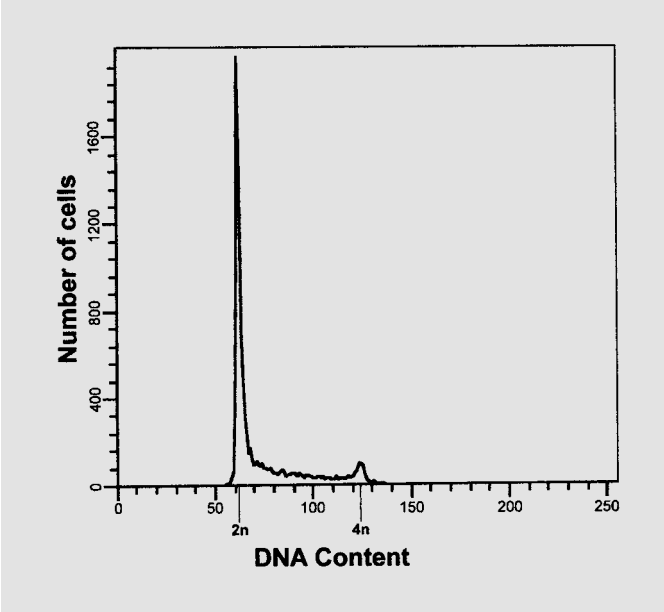
Table 1. Proportion of S phase cells (%) in culture on uncoated (UC), tropocollagen (TC)- and fibrillar collagen (FC)-coated titanium alloy after 24, 48 and 72 h of incubation (SD < 3%).

Incubation time, h	UC	TC	FC
24	24	31	33
48	10	16	13
72	3.5	6	5

should be double that of the first, corresponds to cells with G2/M levels of DNA (4n). Cells scored in the trough have a DNA content between these phases and are considered to represent cells in the S phase. Simultaneously, the cells from each disk were counted by a hemocytometer. Cell counts correlated with the S phase. The results shown in figure 4 compare the average proportion of cells which were found in each of the phases of the cell cycle, after the cells were grown on coated or uncoated titanium alloy for 24 h. There was a higher proportion of G0/G1 cells in the culture on uncoated titanium alloy (73.5%) compared with both the tropocollagen and fibrillar collagen cultures (65 and 63%, respectively; fig. 4). Moreover, this was accompanied by a statistically significant reduction in the proportion of S phase cells on uncoated titanium alloy (24%) compared with both the tropocollagen and fibrillar collagen-coated surfaces (31 and 33%, respectively), as shown in figure 4. The proportion of G2/M cells was very similar in all cultures. These results suggest that culture of the cells on collagen-coated titanium alloy stimulated the cell cycle progression, by accelerating the entry of G0/G1 cells into the S phase. The time course of the proportion of S phase cells in the culture on uncoated and collagen-coated titanium alloy is presented in table 1. The results show an increase in replicating cells on coated titanium alloy compared with those on uncoated titanium alloy following 24, 48 and 72 h of incubation. After 72 h, the proliferation was decreased by confluency of primary osteoblasts on titanium disks.

Expression of Osteopontin in Osteoblasts on Collagen-Coated Titanium Alloy

For studies of osteogenic differentiation, osteopontin was examined, which is expressed early during osteogenesis and at different levels during the maturation of osteoblastic cells [Zohar et al., 1998a, b]. The expression of osteopontin was determined in conjunction with flow



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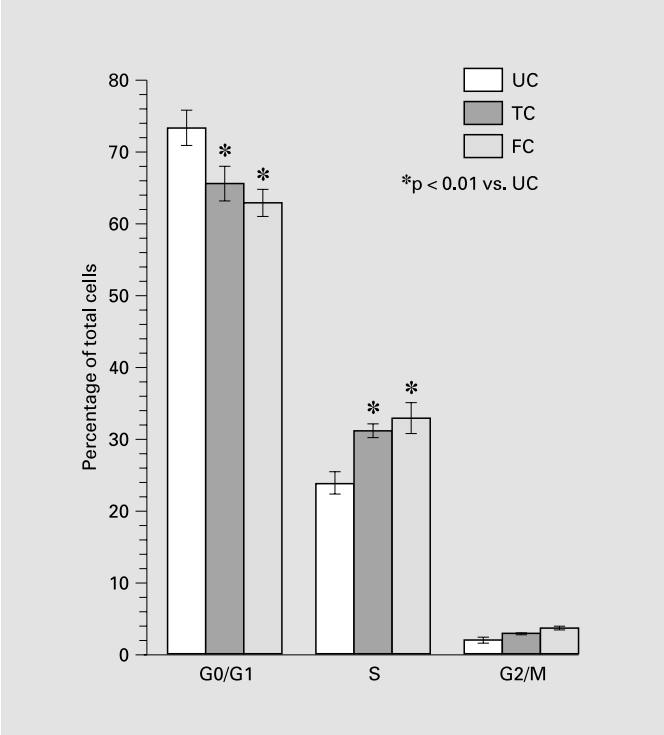

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Fig. 3. A histogram of the DNA content of cells grown on uncoated Ti6A14V disks for 24 h and stained with propidium iodide. In these experiments, the 2n DNA level was approximately 62, while the 4n DNA level in G2/M cells was approximately 124, as predicted.

Fig. 4. The percentage of cells in each phase of the cell cycle after growth on uncoated (UC), tropocollagen-coated (TC), and fibrillar collagen-coated (FC) Ti6A14V disks for 24 h. The cells were stained with propidium iodide. Mean values ± SD obtained with triplicate cultures are represented. The data shown are representative of three separate experiments.

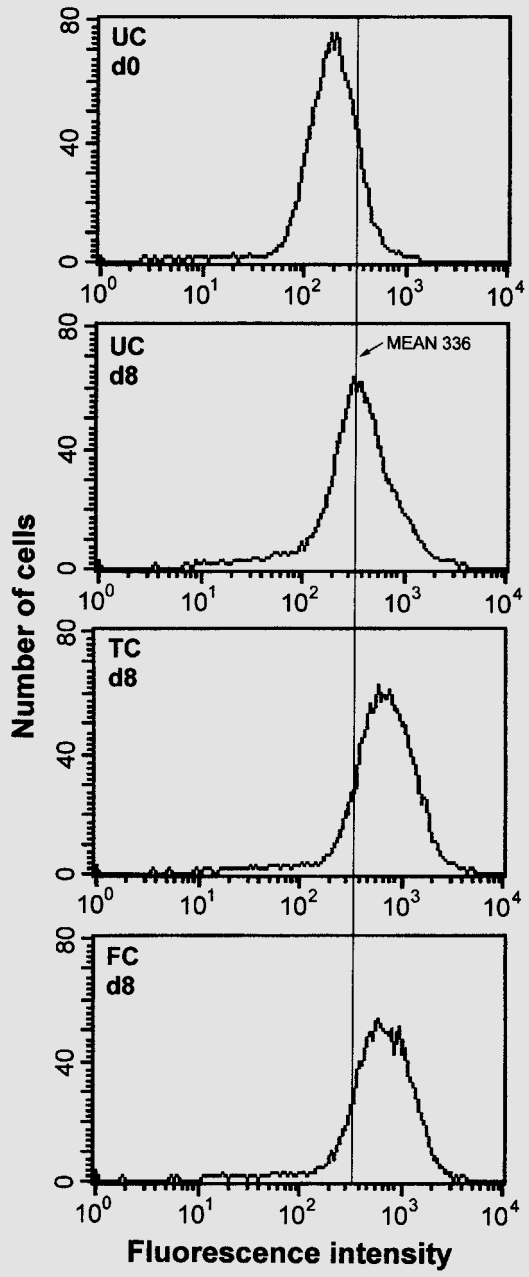

Fig. 5. Fluorescence intensity distributions corresponding to the expression of osteopontin. The vertical line indicates the mean fluorescence intensity of osteopontin in cultures grown on uncoated titanium alloy after 8 days (UC, d8). The position of this line suggests that osteopontin is upregulated in cultures on tropocollagen-coated (TC) and fibrillar collagen-coated (FC) titanium alloy after 8 days. Control cultures at day 0 without differentiation medium (UC, d0) have the lowest mean fluorescence intensity.

cytometry, in order to analyze the events associated with osteogenic differentiation at the single cell level in primary osteoblastic cultures. In this study, osteopontin production was measured after 4, 8, 11 and 18 days of culture. Flow cytometric analysis revealed that osteoblasts adhering to collagen-coated surfaces were capable of producing more osteopontin than cells grown on uncoated titanium alloy (fig. 5). At all times, almost all cells expressed osteopontin. There were no effects of different forms of collagen on osteopontin expression. The difference in osteopontin expression between cells on coated and uncoated titanium alloy was greatest on day 8.

Discussion

Demineralized collagenous bone matrix has the potential to initiate new bone formation locally at the site of implantation [Masi et al., 1992]. Type I collagen is the predominant collagen type in newly formed osteoid and serves as the basis for the mineral scaffold. The potential importance of this matrix protein for the regulation of mineralized tissue homeostasis prompted us to coat the common orthopedic implant metal, titanium alloy, with type I collagen, i.e. with both fibrillar collagen and tropocollagen. This in vitro study investigated the adhesion, proliferation and differentiation of primary osteoblasts on titanium alloy coated with type I collagen.

Cell Adhesion

The adhesion of osteoblasts on titanium alloy is a prerequisite for tissue integration. New bone formation on biomaterials depends on surface structures which promote cell proliferation and the production of extracellular matrix. Important for successful osseointegration of the implant is the adhesion of osteoblasts to the implant surface [Kornu et al., 1996; Puleo et al., 1991; Schneider and Burridge, 1994; Shah et al., 1999; Sinha et al., 1994; Vrouwenvelder et al., 1993].

Our results show that osteoblasts effectively adhere to implants within 2 h in vitro. In terms of cellular kinetics, these data are in agreement with those of Puleo et al. [1991], who found that almost 90% of osteoblasts attached to titanium alloys after 2 h. Puleo and Bizios [1992] also demonstrated that at later times of attachment (after 24 h), focal adhesions formed on titanium alloys.

Our results, however, demonstrate that osteoblasts adhere better on collagen-coated titanium alloy than on uncoated titanium alloy during the first 2 h. When the titanium surfaces were coated with collagen, the osteo-

blasts spread rapidly and formed well-defined focal adhesions and associated stress fibers. There was no difference between the degree of actin reorganization in osteoblasts cultured on fibrillar collagen and tropocollagen. On uncoated titanium alloy, we found that osteoblasts spread more slowly and formed only insignificant focal adhesions and stress fibers during the first 2 h. For osteoblasts, these data support the findings of Cooper et al. [1993], who observed a tenfold greater adhesion rate of osteoblasts to collagen type I than to commercially pure titanium surfaces. The experimental comparison shown by Cooper et al. [1993] suggests that osteoblasts would preferentially bind to collagen type I substrates and implies that the in vivo binding of osteoblasts to commercially pure titanium surfaces is less favorable.

Cytoskeletal organization is important for initial attachment as well as for the stability of the attachment to titanium alloy. Puleo and Bizios [1992] and Sinha et al. [1994] showed a similar sequence of actin filament organization on titanium alloy. Focal contacts have been described as the primary structures for cell-to-substrate adhesion. These structures are important transducers of extracellular signals. Integrins localized to focal contacts are capable of signal transduction [Kornberg et al., 1992, 1991; Sinha and Tuan, 1996; Werb et al., 1989]. The regulation of cellular differentiation and growth may be mediated by the molecules involved in focal contact formation, which in turn is determined by the nature of the substrate. According to Miyamoto et al. [1995], vinculin is one of the last proteins to colocalize to the integrin cluster, and only when integrins have been bound to the extracellular matrix. In conclusion, this phenomenon indicates that a specific cell receptor-collagen type I interaction is a more efficient means of attachment than the undefined process of cell-titanium alloy interaction.

Cell Proliferation

We used flow cytometry to compare the proliferation of osteoblasts cultured on collagen-coated titanium alloy with that of cells grown on uncoated titanium alloy. Cell cycle analysis experiments were carried out to determine whether the collagen coating influenced the proliferation of osteoblasts. Osteoblasts grown both on fibrillar collagen- and tropocollagen-coated titanium alloy had a higher proliferative capacity compared with cells cultured on uncoated titanium alloy. Collagen coating appeared to accelerate the progression of the cells from G0/G1 into the S phase of the cell cycle. We found a lower proportion of G0/G1 cells and a higher proportion of S phase cells on coated surfaces compared with uncoated titanium alloy. These results were consistent with our findings of enhanced spreading of osteoblasts and rapid formation of focal adhesions and their associated stress fibers on collagen-coated titanium alloy. These data support the findings of Masi et al. [1992], who observed that collagen type I gel matrices stimulated proliferation, alkaline phosphatase production and responsiveness to parathyroid hormone in osteoblasts.

Osteoblastic Differentiation

Here, we examined the effect of collagen coating on osteopontin expression by osteoblasts. Using flow cytometry, we analyzed the influence of collagen coating on large numbers of cells with respect to differentiation. When osteoblasts were cultured in the presence of 50 μg/ml ascorbic acid and 10 m*M* β-glycerophosphate, they underwent osteogenic differentiation [Bellows et al., 1986a, b]. Osteopontin is recognized as a marker of osteogenic differentiation [Lopes et al., 1998; Zohar et al., 1998a]. Zohar et al. [1998a] showed that osteopontin is expressed early during osteogenesis and at different levels during the maturation of osteoblastic cells. Flow cytometry is a useful procedure for measuring in vitro the biocompatibility of implant materials for orthopedic and dental surgery [Chauvel-Lebret et al., 1999; Lopes et al., 1998; Prigent et al., 1998; Sumner et al., 1991; Wilke et al., 1998; Zohar et al., 1998a]. In this study, osteoblasts grown on collagen-coated titanium alloy were found to express more osteopontin when compared with the uncoated surface. There were no differences in osteopontin expression between cells grown on fibrillar collagen- and tropocollagen-coated titanium alloy. This observation is consistent with the results for the adhesion and proliferation of cells on fibrillar collagen-coated titanium alloy when compared with tropocollagen-coated surfaces. We therefore conclude that the processing of tropocollagen to fibrillar collagen is not necessary to induce the effects on adhesion, proliferation and differentiation of cells on collagen-coated titanium alloy. Other differentiation-associated markers, including CD44 and alkaline phosphatase activity, also showed an increase in osteoblasts on collagen-coated titanium alloy when compared with the uncoated surface (data not shown). These results suggest that the culture of osteoblasts on type I collagen-coated titanium alloy resulted in a more differentiated cell phenotype. The difference in osteopontin expression between coated and uncoated titanium alloy was maximal on day 8. This result is consistent with previous studies indicating that peak osteopontin levels occur 5–7 days after confluency [Lynch et al., 1995; Zohar et al., 1998a]. Celic et al. [1998]

have previously shown that the expression of mRNA encoding osteopontin and alkaline phosphatase was upregulated in cells cultured on collagen type I, suggesting that interaction with collagen promotes the osteoblastic differentiation of this cell line. Osteoblasts grown on collagen type I exhibited an earlier and enhanced expression of the differentiated phenotype, compared to cells cultured on plastic. Osteopontin was upregulated early, suggesting a role in mediating the attachment of the osteoblasts to collagen [Lynch et al., 1995]. For fibronectin, it was shown that osteopontin mRNA expression was increased three- to fourfold, 24 h after calvaria osteoblast attachment to fibronectin [Carvalho et al., 1998].

In conclusion, type I collagen-coated titanium alloy has been shown here to accelerate the initial adhesion of osteoblasts, promote cell proliferation and enhance the expression of osteopontin. These results suggest that precoating titanium alloy with collagen type I may improve osseointegration. The present findings could be significant for the clinical preparation of titanium alloy implants, because exposure of the coated disks to the cell culture medium for several weeks did not result in a measurable desorption of collagen. Moreover, type I collagen may serve as a suitable carrier for osteotropic factors such as cell adhesion molecules (e.g. fibronectin) or bone-specific growth factors (e.g. bone morphogenetic proteins). Our results further show that the favorable properties of collagen with respect to the adhesion and differentiation of osteoblasts are not lost in the coating and sterilization procedures. Thus, further investigations would need to determine the biocompatibility and osseointegration of titanium alloy in animal experiments. These studies are currently in progress.

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